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| (54) Title: TGF- β FUSION PROTEINS AND THEIR USE IN WOUND HEALING (57) Abstract A transforming growth factor- β fusion protein and a method of preparation transforming growth factor- β fusion protein. The transforming growth factor- β fusion protein comprises a purification tag, at least one proteinase site, an extracellular matrix binding site, and a transforming growth factor active fragment. A method of preparing transforming growth factor- β fusion protein comprises purifying and renaturing transforming growth factor- β protein to provide an active transforming growth factor- β fusion protein preparation. Methods of use of the transforming growth factor- β fusion protein are also provided. | | |

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TGF-beta fusion proteins and their use in wound healing

Background of the Invention

The Transforming Growth Factor beta (TGF β) superfamily is a large group of cytokines that exert profound influences on the physiology of wound healing. Their mode of action in wound healing includes the modulation of stem cell populations, as well as their expression of specific genes that encode matrix proteins, cellular receptors, matrix proteinases and proteinase inhibitors. Numerous animal studies have demonstrated the efficacy of exogenous TGF β in promoting wound healing, which lead to the first clinical applications in the repair of bone, surgical wound healing and in the treatment of diabetic ulcers and burns. Moreover, a single systemic dose of TGF β_1 , given prior to injury (surgery), has been demonstrated to enhance tissue repair and wound healing, suggesting that a single dose, administered systemically before surgery, may improve patient recovery rates.

Clinical studies using TGF β_1 as a therapeutic agent have been hampered by its limited availability. TGF β_1 is usually purified from either human platelets, bone or soft tissues such as placenta and kidney. It is estimated that approximately one ton of bone is required to purify enough TGF β_1 for a single therapeutic treatment. Small amounts of TGF β_1 have been isolated as a recombinant protein which was processed and secreted by transfected mammalian cells into conditioned growth medium. However, the small amounts of TGF β obtained and the high cost of production do not make this a method of production commercially viable.

The potential utility of TGF β_1 as a clinical agent to promote wound healing is complicated by TGF β_1 's potent chemo-attractant and its macrophage and fibroblast activation properties. At elevated levels of TGF β_1 such as occur in chronic fibrotic disorders, especially when local inflammation persists, macrophages and fibroblasts accumulate at the site of the disease. Elevated plasma levels of TGF β_1 has been shown to correlate with a high incidence of hepatic fibrosis, and has also been associated with glomerulosclerosis and pulmonary fibrosis. Therefore, delivery to

and activation of $\text{TGF}\beta_1$ at the site of a wound is desirable for prolonged treatment with $\text{TGF}\beta_1$.

Three distinct $\text{TGF}\beta$ polypeptides have been identified and are designated $\text{TGF}\beta_1$, $\text{TGF}\beta_2$ and $\text{TGF}\beta_3$. The $\text{TGF}\beta$ proteins are expressed as precursor
5 molecules of 380, 442 and 410 amino acids, respectively. These inactive latent $\text{TGF}\beta$ proteins are activated by proteinases such as plasmin, latent $\text{TGF}\beta_1$ binding protein (LTBP) and thrombospondin. The mature form of the proteins are dimers
10 of identical polypeptide chains of 112 amino acids in length. The amino acid sequence of the $\text{TGF}\beta_1$, $\text{TGF}\beta_2$ and $\text{TGF}\beta_3$ polypeptides shows 70 to 80% homology and the sequence conserved in the mature polypeptides includes 9 cysteine residues
15 which determine the inter- and intra-polypeptide disulfide bridge formation in the mature proteins.

LTBP and a 60 kD $\text{TGF}\beta_1$ binding protein appear to mediate the binding of $\text{TGF}\beta_1$ to the extracellular matrix. The close association of $\text{TGF}\beta_1$ with the
15 extracellular matrix possibly maintains the elevated growth factor concentration within the local environment of the healing wound.

The use of $\text{TGF}\beta$ -based medical therapies require the availability of large quantities of pharmaceutical grade $\text{TGF}\beta$ that is free of transmittable hazards
omnipresent in products extracted from animal, in particular human, sources.
20 Therefore, it is desirable to develop a means for preparing large quantities of the mature $\text{TGF}\beta$. It is also desirable that the protein is made from a source which eliminates the possibility of hazardous material contaminating the final product. It is also desirable that the protein is engineered to target specific site where wound
25 healing is desired.

Summary of the Invention

The present invention is directed at a transforming growth factor- β fusion protein, a method of preparation of the transforming growth factor- β fusion protein and methods of using the transforming growth factor- β fusion protein.

30 The transforming growth factor- β fusion protein comprises a purification tag, at least one proteinase site, an extracellular matrix binding site, and a transforming growth factor active fragment.

The method of preparation transforming growth factor- β fusion protein comprises purifying and renaturing transforming growth factor- β protein to provide an active transforming growth factor- β fusion protein preparation.

Methods of use of the transforming growth factor- β fusion protein include
5 methods to reduce surgery recovery time and the preparation of artificial skin.

Brief Description of the Drawings

Features, aspects and advantages of the invention will be more fully understood when considered with respect to the following detailed description,
10 appended claims and accompanying drawing where:

FIG. 1 is a diagrammatic representation of the stages of wound healing observed with TGF β treated collagen matrices;

Detailed Description

15 This invention is directed at genetically engineering TGF β fusion proteins, their expression in *E. coli* and their purification and renaturation of active TGF β . As used herein TGF β , TGF β_1 , TGF β_2 and TGF β_3 , or the active fragment of TGF β , TGF β_1 , TGF β_2 and TGF β_3 means the active portion of TGF β , TGF β_1 , TGF β_2 and TGF β_3 , respectively, present in the mature form of the naturally occurring proteins
20 or other such proteins which exhibit similar biological activity. Also, as used herein, transforming growth factor- β fusion protein means the active portion of TGF β , TGF β_1 , TGF β_2 and TGF β_3 produced in accordance with this invention and may include other regions such as purification tags, as described below. Transforming growth factor- β fusion protein is also intended to mean the active portion of TGF β ,
25 TGF β_1 , TGF β_2 and TGF β_3 which has been cleaved from other regions such as purification tags. The ability to express and renature the active fragment of TGF β , in the absence of the pro-region, in accordance with the present invention, into a biologically active dimer is a surprising result. Other workers in the field have concluded that their experiments demonstrate that the pro-region of TGF β is essential
30 for the folding and assembly of TGF β dimers. Therefore, in view of the teaching in this field expression of the active portion of TGF β , in the absence of the pro-region, would not be expected to result in a biologically active dimer.

The present invention is also directed at the use of these proteins in the treatment of wound healing.

In accordance with this invention a prokaryotic expression vector is engineered to produce a series of fusion proteins which comprise a cDNA sequence encoding the active fragment of human $TGF\beta_1$, $TGF\beta_2$, or $TGF\beta_3$, by methods well known to those skilled in the art. Additionally, the fusion proteins may comprise a purification tag, proteinase-sensitive linker sites and binding domain such that the protein sequence may contain all or some of the following elements:

purification tag:proteinase site:ECM binding site:proteinase site: $TGF\beta$

10 The inclusion of a purification tag facilitates purification of the fusion protein. A first proteinase site is included to permit cleavage and release of the purification tag after purification of the fusion protein, if desired.

The ECM binding site facilitates delivery of the fusion protein to the desired site of action. The ECM binding site is, therefore, chosen to direct the $TGF\beta$ to the site to be healed. Deliver of the $TGF\beta$ to the site to be treated reduces the amount of $TGF\beta$ required to be administered to be effective and reduces the concentration of circulating $TGF\beta$ which may result in undesirable side effects.

In some circumstances it is also desirable to included a second proteinase binding site, which may be the same as or different from the first proteinase site.

20 Where the second proteinase site is different from the first proteinase site, the second proteinase site allows the $TGF\beta$ to be released from the ECM binding site once it has reached its site of action. The proteolysis occurs as a result of the action of proteinases released at the site of the injury. Since the release of the proteinases is over a period of time, as the healing process proceeds, the $TGF\beta$ is also released slowly, over a period of time. Therefore, a second proteinase inhibitor is used where such "time release" is desirable. In applications where release of the $TGF\beta$ is undesirable, the second proteinase site is omitted.

In one embodiment of the present invention extracellular matrix (ECM) binding domains are used which are selective for either collagen ($TGF\beta$ -F2), fibronectin ($TGF\beta$ -F3) or cell surface. In one embodiment of the present invention the sequence selective for collagen was modified (collagen^m), from the naturally occurring sequence of:

30

Trp-Arg-Glu-Pro-Ser-Phe-Cys-Ala-Leu

to:

Trp-Arg-Glu-Pro-Ser-Phe-Met-Ala-Leu

to ensure that the Cys would not interfere with the refolding/renaturation of the

5 TGF β .

Illustrative combinations of fusions proteins suitable for use in the present invention are summarized in Table I. The list in Table I is intended to illustrate the types of fusion proteins which are intended by the present invention and are not intended to limit the scope of the invention. Those skilled in the art will be aware
10 that practice of the present invention could include other elements such as other purification tags, such as epitope tags and specific binding proteins and enzymes, other proteinase sites, such as thrombin cleavage site, factor Xa cleavage site, plasmin cleavage site, chymotrypsin cleavage site, elastase cleavage site, trypsin cleavage site, pepsin cleavage site, thermolysin cleavage site, other binding
15 sequences such as cell surface and tissue specific antigens, and other TGF β 's fragments could be substituted for TGF β_1 . One skilled in the art will also be aware that modifications of the sequences of these elements could also be used which would not change the functional properties for which they are used. Those skilled in the art will also realize that "linkers" could be added between the elements, to facilitate
20 cloning and manipulation of the resultant clones without changing the functional properties of the resultant fusion proteins.

Table I

| Tag | proteinase site | Binding domain | proteinase site | TGF β | SEQ ID NO. |
|-----------------------|-----------------|-----------------------|-----------------|--------------------------|----------------|
| (His) ₆ | none | none | none | TGF β ₁ | 22:30 |
| (His) ₆ | thrombin | none | none | TGF β ₁ | 22:15:30 |
| 5 (His) ₆ | thrombin | collagen ^a | none | TGF β ₁ | 22:15:20:30 |
| (His) ₆ | thrombin | collagen ^a | thrombin | TGF β ₁ | 22:15:20:15:30 |
| (His) ₆ | thrombin | collagen ^a | factor Xa | TGF β ₁ | 22:15:20:13:30 |
| (His) ₆ | factor Xa | none | none | TGF β ₁ | 22:13:30 |
| (His) ₆ | factor Xa | collagen ^a | none | TGF β ₁ | 22:13:20:30 |
| 10 (His) ₆ | factor Xa | collagen ^a | factor Xa | TGF β ₁ | 22:13:20:13:30 |
| (His) ₆ | factor Xa | collagen ^a | thrombin | TGF β ₁ | 22:13:20:15:30 |
| (His) ₆ | thrombin | fibronectin | none | TGF β ₁ | 22:15:18:30 |
| (His) ₆ | thrombin | fibronectin | thrombin | TGF β ₁ | 22:15:18:15:30 |
| (His) ₆ | thrombin | fibronectin | factor Xa | TGF β ₁ | 22:15:18:13:30 |
| 15 (His) ₆ | factor Xa | fibronectin | none | TGF β ₁ | 22:13:18:30 |
| (His) ₆ | factor Xa | fibronectin | factor Xa | TGF β ₁ | 22:13:18:13:30 |
| (His) ₆ | factor Xa | fibronectin | thrombin | TGF β ₁ | 22:13:18:15:30 |

20 In the practice of the present invention, fusion protein expression vectors are expressed in *E. coli* or other suitable hosts and are isolated and purified. The proteins of the present invention expressed in bacteria accumulate in inclusion bodies in a precipitated form. In such cases it is desirable to solubilize the protein in a denaturing, or other suitable buffer for further purification. Such denaturing buffers include a denaturing agent such as 8 M urea and may also include reducing agents such as dithiothreitol (DTT) or β -mercaptoethanol.

In one embodiment of the present invention the purification of the fusion proteins uses a purification tag comprising polyhistidine expressed as an N-terminal portion of the fusion protein. When the purification tag comprises polyhistidine, a metal chelate binding medium such as nickel chelate medium is used as the purification medium. In other embodiments of the present invention the purification tag comprises epitopes, schistosoma japonicum glutathione S transferase (GST)ribonuclease S or Hemagglutinin, and the binding medium comprises PBS with an eluting agent such as low pH, peptides or glutathione. Other purification tags, their associated binding media and suitable conditions for binding and eluting the proteins from the media are summarized in Table II.

Table II

| Tag | Binding Medium | Binding Buffer | Elution Buffer |
|--------------------|-------------------|---------------------------|-----------------------------|
| (His) ₆ | metal chelate | denaturing buffer, pH 6.5 | denaturing buffer, pH 4.0 |
| Ribonuclease S | S-protein agarose | PBS | PBS + low pH or peptides |
| 5 GST | affinity medium | PBS | PBS + low pH or glutathione |
| Hemagglutinin A | Immuno-affinity | PBS | PBS + low pH or peptides |

Proteins are isolated from host cells transformed with TGF β fusion protein expression vector and proteins are solubilized in a denaturation buffer adjusted to a pH of about pH 8.0. Suitable denaturation buffers are comprise a high concentration of a denaturant such as 8 M urea. Such buffers may also comprise a reducing agent such as β -mercaptoethanol. Any particulate material in the solubilized protein sample is removed by centrifugation at about 20,000 x g for about 20 minutes.

The supernatant, which includes TGF-fusion protein, is collected and mixed with a metal chelate medium such as Ni-NTA resin and gently agitated for about 1 hour. The TGF-fusion protein/resin mixture is then loaded onto a column, the resin is allowed to settle and the liquid to drain off. The protein/resin mixture is washed with denaturation buffer adjusted to a pH of about 8.0 to remove non-specifically bound proteins. Additional non-specifically bound proteins are eluted by washing the protein/resin mixture with denaturation buffer, adjusted to pH of about 6.5. The TGF-fusion protein is then eluted from the metal chelate medium by washing with denaturation buffer adjust to a pH of about 4.0 and the eluate proteins, which include TGF β , are collected.

The eluate is diluted to a protein concentration of about 0.05 to about 0.5 mg/ml with denaturation buffer, adjusted to a pH of about 8.0. The diluted protein sample is then further diluted with about 4 volumes of freshly made buffer such as about 20 mM Tris-HCl, pH 8.0, about 250 mM NaCl, about 0.05% (v/v) NP-40, about 2 mM reduced glutathione and about 0.2 mM oxidized glutathione.

The diluted protein is sealed in a container and stored overnight at about 4°C. The diluted protein is then dialyzed against an equal volume of a dialysis buffer such as about 20 mM Tris, pH 8.0, about 250 mM NaCl and about 20% (v/v) glycerol for about 20 minutes. After about 20 minutes, and then about every 20 minutes thereafter, the dialysis buffer is replaced with twice the volume of dialysis buffer previously used, until the final volume is about 10 times the volume of the dialysate. The dialysis is then stored overnight at about 4°C without stirring. The next morning the dialysis is stirred for about 30 minutes. The dialysis buffer is then replaced and the dialysis is stirred for about 2 hours. The contents of the dialysis bag is then collected and any particulate matter is removed by centrifugation at about 5,000 rpm for about 20 minutes at 4°C.

The TGF β fusion protein isolated, purified and renatured as described above, exhibits an antiproliferative activity comparable to TGF β_1 controls (naturally occurring TGF β_1).

15 The present invention is also directed at the use of TGF β fusion proteins in wound healing.

It is desirable to administer the TGF β of the present invention as a preventative measure prior to surgery. In such cases, TGF β prepared in accordance with the process of the present invention, is administered as a single dose of 100 to 20 500 μ g/ml/kg body weight, intravenously, about 24 hours prior to surgery.

The present invention is also directed at a mesenchymal stem cell trap. FIG. 1 is a diagram of wound healing stages observed within TGF β treated collagen matrices. Depicted are three major features: (I) recruitment and expansion of a mesenchymal stem cell (MSC) population, (II) elaboration (of factors) and 25 differentiation of cellular phenotype and (III) resolution and remodeling of the extracellular matrix. TGF β impregnated collagen matrices are utilized to selectively reinforce the proliferation of mesenchymal stem cells that are present in low abundance within human bone marrow aspirates under conditions where the remainder of the cellular components of the marrow do not survive. Rescue and 30 selection of TGF β responsive stem cells from human bone marrow aspirates is performed after about 15 days of serum deprivation. Serum deprivation results in the death of unwanted cells.

The present invention is also directed at the use of genetically engineered TGF β fusion proteins, produced in prokaryotes, for therapeutic advantage in the clinical management of *ex vivo* histogenesis, the preparation of "Artificial Skin" and surgical wound healing. Collagen matrices and sheets which are currently used as a "skin or tissue replacement", though optimal in terms of structural integrity and biodegradability, are highly antigenic in wound healing applications, resulting in inflammatory responses (rejection) and fibrosis (scarring). In contrast, TGF β impregnated collagen matrices inhibit inflammatory processes while promoting angiogenesis and histogenesis. TGF β is a natural and critical component regulating epithelial-mesenchymal interactions in the developmental morphogenesis of skin appendages. Collagen bound TGF β -F2 fusion proteins function effectively to select and expand (capture) a population of mesenchymal stem cells *in vitro*.

An autologous "artificial" skin is prepared by selecting and expanding a population of explanted human fibroblasts, along with other resident mesenchymal precursors, within TGF β impregnated collagen sheets. This procedure is continued *in vitro* up to an optimized point whereby the collagen sheet is effectively cellularized yet not degraded. At or just prior to this point, the collagen/connective tissue sheet is epithelialized by the application of an explanted plug of keratinocytes.

The human artificial skin comprised of TGF β impregnated matrix such as collagen sheets is cellularized and epithelialized in a 2-stage process:

1. Enrichment (recruitment and expansion) of pluripotent stem cells facilitate normal histogenesis and wound healing. Recombinant TGF β fusion proteins is applied to the cellularized "skin" (i.e., cellularized/epithelialized collagen sheets) and/or the wound surface.
2. Secondary application of TGF β fusion proteins inhibits rejection and promotes fusion of cultured tissues. The timing of each stage of the *ex vivo* tissue culture, as well as the thickness and physiochemistry of the collagen sheets, are determined by visual observation. The TGF β fusion proteins play a pivotal role in promoting normal skin healing while suppressing the inflammatory responses and granulation tissue associated with chronic wounds.

Example 1Recombinant Constructs and Protein Expression

Cytoplasmic RNAs isolated from EW-1 Ewing's sarcoma cells and human MG-63 osteosarcoma cells were reverse transcribed into first-strand cDNA using an antisense oligonucleotide primer, by methods well known to those skilled in the art. PCR amplification was performed on the first strand cDNA and the resulting PCR products were separated electrophoretically, by methods well known to those skilled in the art. Visualized bands were purified from agarose gel by GeneClean (Bio 101) and ligated to a TA vector (Invitrogen). Color-selected clones were isolated and analyzed by restriction mapping, followed by nucleotide sequence determination.

To construct a tripartite fusion protein the cDNA sequence encoding the C-terminal 112 amino acids of human $TGF\beta_1$, obtained by RT-PCR, was ligated in frame to pET28 vector (Novagen), and maintained in the XL Blue strain of *E. coli* BL21(DE3). The orientation and reading frame of the insert was confirmed by DNA sequence analysis.

Each of the pET- $TGF\beta_1$ constructs were transformed into *E. coli* BL21(DE3), and high level expression of recombinant proteins was induced in the presence of 0.4 mM isopropyl thiogalactopyranoside (IPTG) for 5 hours at 37°C with shaking at 300 rpm. The first construct, pET- $TGF\beta_1$ -F1, contained a (His)₆ leader sequence at the N-terminus of the fusion protein, a thrombin cleavage site in the first proteinase site, followed by a truncated active $TGF\beta_1$ fragment (i.e. the plasmid encoded SEQ ID NOs. 22:15:30). Two additional constructs, pET- $TGF\beta_1$ -F2 and pET- $TGF\beta_1$ -F3, incorporating collagen-binding and fibronectin-binding sites, respectively (i.e. the plasmids encoded SEQ ID NOs. 22:15:20:30 and SEQ ID NOs. 22:15:18:30, respectively), were designed for extracellular matrix targeting of these fusion proteins.

Example 2Small Scale Induction of Recombinant $TGF\beta$
Fusion Proteins in *E. coli*

E. coli, BL21(DE3), transformed with pET- $TGF\beta_1$ -F1, prepared as described above, with a protein tag of 6 Histidine residues at the N-terminal of the fusion

protein were inoculated into 5 ml of 2 x YT medium in the presence of kanamycin (50 μ g/ml kanamycin sulfate, supplied by GIBCO-BRL). The cultures were incubated at 37°C with shaking (225 rpm; Lab-Line orbital shaker) until visible bacterial growth was observed, about 2 to 3 hours.

- 5 Three ml of cell suspension was transferred to 12 ml of YT medium which included 50 μ g/ml kanamycin sulfate. The cultures were incubated at 37°C with shaking (225 rpm) until visible bacterial growth was observed, about 2 to 3 hours. The cultures were then monitored at A_{600} . When the cultures reached A_{600} of 0.6 to 0.8 the expression of the fusion protein was induced by the addition of IPTG (Sigma
10 I-6758) to a final concentration of 0.4 mM. The cultures were incubated at 37°C with shaking at 300 rpm.

- The remaining 2 ml of the original culture, the pre-induction samples, were centrifuged in a microfuge (Eppendorf 5415C) at 10,000 rpm for 2 minute and the supernatant aspirated. Two hundred μ l of SDS sample buffer (reducing) was added
15 to the cell pellet and the sample was mixed and heated at 95°C to 98°C for 7 minutes. The pre-induced samples were stored at -20°C until needed.

- After 3.5 to 4 hours of incubation in the presence of IPTG, 1 ml aliquots of the cultures were removed and centrifuged as described above for the uninduced samples. The cell pellet was dissolved in 300 μ l SDS sample buffer (reducing) and
20 heat at 95°C to 98°C for 7 minute. The induced samples were stored at -20°C until needed.

The remainder of the cells were collected by centrifugation at 2,000 to 3,000 rpm for 10 minute. The cell pellets were frozen at -20°C until needed.

25

Example 3

Gel Electrophoresis and Protein Staining

- Ten μ l of each of the pre-induction and induced samples prepared as described in Example 2, were loaded, per lane, on a 8 to 16% gradient gel (1.5 mm thick). Five to 10 μ l of protein standard (e.g., Novex) were included on the gel in
30 separate lanes. The gels were run in standard SDS gel electrophoresis buffer at 100 to 125 volts for 2 to 2.5 hours until the dye front reached the bottom of the gel. The gel was fixed in 40% (v/v) methanol, 10% (v/v) acetic acid for 20 minutes with

gentle shaking. The gel was then stained with 0.25% (w/v) Coomassie blue stain (in 50% (v/v) methanol, 10% (v/v) acetic acid) for 20 minutes. The gel was then destain twice (1 to 2 hours each time) in 40% (v/v) methanol, 10% (v/v) acetic acid. The gels were then viewed over a light box to determine, qualitatively, the level of
5 induction.

Finally, the gel was destained overnight in 10% (v/v) methanol, 10% (v/v) acetic acid, dried and stored.

Example 4

10 Small Scale Induction of Recombinant TGF β Fusion Proteins in *E. coli*

A clone with a high induction response determined by the process of Examples 1 to 3 was selected and 200 μ l of a 3 to 4 hour culture was added to 20 ml of medium supplemented with 50 μ g/ml kanamycin. The culture was incubated
15 at 37°C with shaking at 225 rpm overnight. Ten ml of the overnight culture was inoculated into 500 ml medium supplemented with 50 μ g/ml kanamycin. The A_{600} of the cells was monitored. When the A_{600} reached 0.7 to 0.8, 1 ml aliquots were collected, centrifuged and the cell pellet denatured as described in Example 2. This sample represented the pre-induction sample.

20 Five hundred μ l IPTG was added to the culture and the culture was incubated with shaking at 275 rpm for 4 to 5 hours. A 1 ml aliquot was collected for the induced sample.

Cells in the remaining culture were collected by centrifugation at 4°C at 8,000 rpm (7,500 x g) for 15 minute. The supernatant was decanted and the cell
25 pellets were stored at -20°C.

Induction of the pET-TGF β_1 -F1 fusion protein in the BL21(DE3) strain of *E. coli* in the presence of IPTG resulted in high yield expression of the 12.5 kD His-tagged C-terminal active fragment of TGF β_1 . The vast majority of the expressed protein was found sequestered in insoluble inclusion bodies in the *E. coli*. The
30 expressed protein was recovered by solubilization in 8 M urea followed by Ni-NTA chelate chromatography.

Example 5Renaturation of Recombinant TGF β_1 -F1

Solubilization and refolding of recombinant TGF β_1 -F1 fusion protein was performed under a variety of experimental conditions.

5 Method I: A single step method used low concentrations of urea and DTT.

Method II: A redox system used DTT in conjunction with glutathione.

Method III: A modification of the glutathione redox system involved a slow dilution of the urea-solubilized material (solubilized in 10 mM Tris base, 100 mM
10 Na₂HPO₄, and 8 M urea, pH 8.0) with a balanced redox buffer (2 mM reduced glutathione, 0.2 mM oxidized glutathione, 20 mM Tris-HCl, pH 8, 250 mM NaCl, 0.05% NP-40; NP-40 supplied by Sigma N0896 for 48 hours prior to dialysis).

In each case, the renatured preparation was dialyzed in protracted steps against Tris buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.05% NP-40, and
15 10% glycerol), and further purified by nickel chelate chromatography on a Ni-NTA (Qiagen) medium. Non-specifically bound proteins were removed from the protein bound medium by washing with 20 mM Tris-HCl buffer, pH 8, containing 25 mM imidazole, 500 mM NaCl, and 10% glycerol. After non-specifically bound proteins were removed from the medium the bound fusion protein was eluted with 20 mM
20 Tris-HCl buffer, pH 8, containing 500 mM imidazole, 500 mM NaCl, and 10% glycerol.

Attempts were made to refold TGF β_1 -F1 using various refolding schemes which had previously been successful with renaturing other proteins.

Solubilizing the inclusion bodies in 2 M urea and 2 mM DTT (Method I) was
25 effective in renaturing other proteins (IGF-1: Chang *et al.* In: *Protein Folding In Vivo and in Vitro*, ed by Cleland, J.L., Symposium Series 526, American Chemical Society, Washington DC, pp. 178-188, 1993, which is incorporated herein by reference) but did not produce an appreciable amount of biologically active TGF β_1 .

Refolding/reoxidation of TGF β_1 -F1 in the presence of glutathione and DTT
30 as described by Glocker *et al.* (*J. Biol. Chem.* 91, 5868-5872, 1994; Method II, which is incorporated herein by reference) yielded a very small amount of renatured TGF β_1 with little biological activity.

Method III, refolding the urea-solubilized aggregates from inclusion bodies at a protein concentration of 0.1 mg/ml in an optimized glutathione redox couple system (2 mM reduced: 0.2 mM oxidized) was determined to be the most effective method for renaturation of the isolated TGF β .

- 5 Renaturation of the TGF β_1 fusion protein (~13 kD) into soluble homodimers (~30 kD) was demonstrated by SDS-PAGE performed under non-reducing conditions.

Biological activity of the renatured recombinant TGF β_1 fusion protein was confirmed by an Mv1Lu cell proliferation assay (Example 7) in which purified
10 human platelet TGF β_1 served as a positive control. Dialyzed protein fractions (5 to 30 μ g/ml) from the glutathione redox-couple refolding system (Method III) exhibited an anti-proliferative activity comparable to the TGF β_1 control treatments (50 to 200 pg/ml) in Mv1Lu cells. In contrast, fractions from Methods I and II, respectively, exhibited little or no biological activity. The inability of these fractions
15 to inhibit Mv1Lu cell proliferation indicated that there was no effect of the final dialysis buffer components, at these dilutions, on Mv1Lu cell proliferation activity.

The biological activity of renatured TGF β_1 in solution, and when it was adsorbed onto collagen- or fibronectin-coated surfaces were examined. In solution, renatured recombinant TGF β_1 (by Method III) exhibited an anti-proliferative effect
20 on Mv1Lu cells. Mv1Lu cells are highly efficient at adhesion and proliferation on collagen- or fibronectin-coated surfaces. TGF β_1 adsorbed onto collagen and/or fibronectin exerts a similarly potent antiproliferative effect on Mv1Lu cells (~70% on collagen and ~40% on fibronectin) without affecting the ability of these cells to adhere to these surfaces.

Example 6Extraction, Purification and Renaturation of
Biologically Active TGF β Fusion Proteins in High Yield
from *E. coli* Inclusion Bodies

5 Frozen *E. coli* pellets, transformed with TGF β_1 -F1 or TGF β_1 -F2 of induced culture, prepared as described in Example 4, were suspended in 25 ml of lysis buffer (20 mM Tris, pH 8.0, 250 mM NaCl, 0.05 % (v/v) NP-40, 0.4 mM PMSF, 25 μ l β -mercaptoethanol, 10 mg lysozyme) at 4°C, and incubated at 4°C for 30 minutes with constant stirring. The suspension was transferred to 50 ml centrifuge tubes and
10 sonicated with a Polytron-sonicate, at a setting of #7 or 25,000 rpm (two cycles of 30 seconds) at 4°C. The sonicated lysate was then centrifuged at 12,000 x g at 4°C for 20 minutes. The supernatant was decanted and the pellet, which included inclusion bodies, were washed with Basic Binding buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.05 % (v/v) NP-40) at 4°C and centrifuged at 12,000 x g for 20
15 minutes.

The pellet, which included inclusion bodies, was suspended in 25 ml of denaturation buffer (8 M urea, 0.1 M sodium phosphate, 10 mM Tris, pH 8.0, made fresh daily by passing a stock 8 M urea solution (480 g/l of warm dH₂O) through a Mix-bed resin (TMD-8, Sigma) just before use and prior to the addition of the
20 buffers. Seventeen μ l of β -mercaptoethanol was added and the solution was vortexed vigorously until most of the pellet was dissolved.

The solution was then centrifuged at 20,000 x g for 20 minutes at room temperature and the supernatant was collected.

About 5 μ l of the supernatant was aliquoted into a microfuge tube, and 795
25 μ l of water and 200 μ l of BioRad Protein Assay Reagent was added, mixed well and the samples were read at 595 nm in a spectrophotometer using 5 μ l of denaturation buffer, 790 μ l of water and 200 μ l of BioRad Protein Assay Reagent as a blank. The protein concentration was estimated from a protein standard curve.

The supernatant, which included the TGF fusion protein, was mixed with
30 Ni-NTA resin (5 ml bed volume, equilibrated with denaturation buffer) in a 50 ml centrifuge tube and rocked for 1 hour at room temperature. The TGF fusion protein/resin mixture was loaded onto a 25 ml column, and the resin was allowed to

settle and the liquid to drain off. The TGF-fusion protein/resin was washed with 40 ml of denaturation buffer, pH 8.0.

The TGF-fusion protein/resin was then washed with 30 ml of Ni-chelating column wash buffer A (denaturation buffer, adjusted to pH 6.5 with 2 M HCl, made fresh daily). The TGF-fusion protein was eluted from the resin with Ni-chelating column Elution buffer B (denaturation buffer adjusted to pH 4.0 with 2 M HCl, made fresh daily). One ml fractions were collected. The protein concentration of 5 μ l of each fraction was determined as described above.

Fractions with protein readings greater than 0.1 mg/ml were pooled. The volume was measured and the protein concentration of the pooled fractions was determined as described above.

The pooled fractions were diluted with denaturation buffer, pH 8.0 to a final protein concentration of <0.5 mg/ml (<0.1 mg/ml for F2 constructs). The pooled samples were then further diluted with 4 volumes of freshly made Redox Buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.05% (v/v) NP-40, 2 mM reduced glutathione, 0.2 mM oxidized glutathione) dropwise on ice with vigorous mixing.

The diluted protein was sealed in a container and stored overnight at 4°C. The diluted protein was then dialyzed against an equal volume of dialysis buffer (20 mM Tris, pH 8.0, 250 mM NaCl, 20% (v/v) glycerol) for 20 minutes. After 20 minutes, and every 20 minutes thereafter, the dialysis buffer was replaced with twice the volume of dialysis buffer previously used, until the volume of the dialysis buffer was 1 liter. The dialysis was then stored overnight at about 4°C without stirring. The next morning the dialysis was stirred for 30 minutes. The dialysis buffer was replaced and the dialysis was stirred for 2 hours. The contents of the dialysis bag was collected, centrifuged at 5,000 rpm for 20 minutes at 4°C and the supernatant was collected. The protein concentration of the collected supernatant was determined and the supernatant was stored at -70°C.

Induction of the TGF β_1 -F2 fusion protein in the BL21(DE3) strain of *E. coli* yielded high levels of expression of the 12.5 kD polypeptide which was found in inclusion bodies. The inclusion bodies were solubilized in 8 M urea and purified by Ni-NTA metal chelate chromatography. Similar to the TGF β_1 -F1 fusion protein, pure TGF β_1 -F2 protein was extracted, as demonstrated by a single 12.5 kD band on

SDS-PAGE, in the presence of DTT. Upon oxidative refolding and dialysis, approximately 15 mg of pure soluble protein was obtained from a 250 ml bacterial culture.

To evaluate the kinetics of refolding, samples were taken at specific time intervals after the initiation of refolding and iodoacetamide (50 mM) was added immediately to block further reaction of the sulfhydryl groups. The samples were stored at 4°C until analyzed by non-reducing SDS-PAGE. Dimers began to form after 2 hours of refolding, reaching maximal levels at 4 to 10 hours under the conditions described above.

Renaturation and assembly of the TGF β_1 -F2 polypeptide (12.5 kD) into active soluble homodimers (25 kD) was demonstrated in the Mv1Lu cell proliferation assay in which commercially available purified human platelet TGF β_1 served as a positive control. The TGF β_1 -F2 fusion protein renatured from bacterial inclusion bodies by the glutathione redox refolding method exhibited an anti-proliferative activity comparable to the TGF β_1 control treatments, although the specific activity of the recombinant growth factor preparation was considerably (~300 times) lower. Renatured TGF β_1 -F2 fusion protein bound to collagen-coated wells also inhibited the proliferation of Mv1Lu cells. However, the level of inhibition observed for collagen-bound growth factor was much smaller than that observed when the unbound TGF β_1 -F2 protein was added directly to the culture medium.

Example 7

Bioassay for TGF β Antiproliferative Effect on Mink Lung Epithelial-Like Cells (mv1lu)

Mink lung epithelial-like cells (Mv1Lu; American Type Culture Collection No. ATCC CCL64) were grown to a subconfluent density (80%) in DMEM (GIBCO) with 10% (v/v) fetal calf serum (FCS). The medium also contained Gentamicin (50 μ g/ml) and Fungizone (20 μ g/ml). The cells were harvested by trypsinizing, in 0.25% (w/v) trypsin, 1 mM EDTA in Hanks BSS (Gibco/BRL) and plated in a 24 well flat-bottomed cell culture plates, with 100 to 150 x 10³ cells/well, in DMEM with 10% (v/v) FCS.

Positive control assays included human recombinant TGF β in assay medium (0.1% (v/v) FCS in DMEM with 2 to 250 pg TGF β /ml). Negative controls included no human recombinant TGF β (0 TGF β /ml) and dialysis buffer was added instead of TGF β .

- 5 Samples were serially diluted into the assay medium. Cells were rinsed twice with DMEM and assay media was added (500 μ l/well for controls and testing samples). The plates were incubated at 37°C with 5% (v/v) CO₂ for 20 hours.

- ³H-Thymidine (ICN, Cat. # 2407005, 1 mCi/ml) uptake assays were performed by adding 20 μ l/well (1 μ Ci) of ³H-thymidine and the incubation was
10 continued for an additional 4 hours.

- The media was removed and discarded. The wells were washed, three times, each for 10 minutes with 1.5 ml of cold 10% (w/v) trichloroacetic acid (TCA) and washed once with water. Five hundred μ l of 0.2 M NaOH was added to each well and the samples were incubated at 37°C for 1 hour. The samples were checked
15 visually to determine if the cells were solubilized. If they were not, they were further incubated at 37°C until solubilized.

- At the end of the solubilization the samples were mixed gently and 200 μ l of the contents of each well was added to a scintillation vial. An equal volume of 0.5 M acetic acid was then added to each sample and the samples were mixed.
20 Scintillant (Ready Safe) was added to each of the samples and they were count for two minutes in a scintillation counter. After storage in the dark overnight the samples were again counted for 2 minutes.

Example 8Epithelialization of Collagen Gels with
Human Keratinocyte Buttons

TGF β treated collagen carrier stem cell trap was prepared as follows: TGF β_1 (20 ng/ml) and TGF β_1 -F2 fusion protein (25 μ g/ml) were reconstituted in DMEM and 200 μ l of TGF β_1 or TGF β_1 -F2 was added to collagen carrier immobilized in 24 well cell culture plates. The samples were incubated for 2 hours at 22°C. At the end of the incubation, the wells, which included the samples, were rinsed twice with 1 ml each of DMEM. The DMEM medium also included Gentamicin (50 μ g/ml) and Fungizone (20 μ g/ml).

Dermal fibroblasts/mesenchymal cells were harvested as follows: Fresh human skin from surgery was collected and rinsed twice with sterile phosphate buffered saline (PBS). The adipose tissue was excised and the skin was cut into 4 mm x 10 mm samples. The skin samples were incubated in prewarmed 0.5% (w/v) dispase/DMEM (10 ml for 2 x 2 cm² skin) at 37°C for 90 minutes with agitation. At the end of the incubation, the keratinocyte layer was peeled off using fine forceps and stored on ice cold DMEM until required.

The dermis was incubated in collagenase-dispase solution (0.5% (w/v) dispase and 1,000 units/ml collagenase) in PBS for an additional 2 hours with agitation (10 ml for 2 x 2 cm² skin). At the end of the incubation, the collagenase/dispace was neutralized by the addition of FCS to a final concentration of 10% (v/v). The samples were then gently homogenized by drawing the mixture into a pipette and releasing it 20 times. The mixture was then filtered through a 100 μ mesh filter and centrifuged to collect cells.

The cells were resuspended in 0.5% (v/v) FCS/DMEM and plated at 200 x 10³ cells/ml/well/collagen carrier. The plates were then incubated at 37°C with 5% (v/v) CO₂ overnight.

At the end of the overnight incubation the medium was replaced with fresh 0.5% (v/v) FCS/DMEM. The plates were then incubated for an additional 1 to 3 days.

Keratinocyte buttons were prepared as follows: Fresh keratinocyte sheet from human skin was harvested, as described above. The keratinocyte sheet was cut into

2 x 2 mm² buttons. Medium was removed from each of the above described wells and the wells were rinsed once with DMEM. The keratinocyte buttons were implanted on the collagen carrier, immobilized with 3 μ l of neutralized collagen (Vitrogen 1 mg/ml, neutralized with 10 X PBS and 0.1 M NaOH, pH 7.2) and incubated at 37°C for 30 minutes. At the end of the incubation, 1 ml of keratinocyte serum free medium (SFM, Gibco/BRL Cat# 17005-042) was added to each well and the samples were incubated at 37°C with 5% (v/v) CO₂. The medium was replaced every 3 days.

Keratinocyte outgrowth assays were conducted as follows: The culture media was removed from the wells by suctioning, and the samples were fixed in 3% (v/v) paraformaldehyde/PBS for 30 minutes at 22°C. The fixative was then removed from each well, and the samples were stained with 0.5% (w/v) Nile Blue Sulfate/1% (v/v) sulfuric acid/PBS for 1 hour at 37°C. The keratinocyte outgrowth was photographed with Polaroid 55 films.

15

Example 9

Cell Proliferation and Matrix Binding Assays

The biological activity of solubilized recombinant TGF β ₁-F1 was tested in a series of cell proliferation assays as described by Ikeda *et al.* (*Biochemistry* 26, 2406-2410, 1987, which is incorporated herein by reference) using Mink Lung epithelial (Mv1Lu) cells with minor modifications.

Mv1Lu cells were maintained in log-phase growth in Dulbecco's modified Eagles medium (DMEM; GIBCO) supplemented with 1% penicillin/streptomycin and 10% (v/v) fetal calf serum (FCS; GIBCO) at 37°C, 5% (v/v) CO₂ in humidified air. For cell proliferation assays, cells were seeded in 24-well plates (Costar) at a density of 1.5 x 10⁵ cells/well in normal growth medium. After an overnight incubation, the medium was replaced with 0.1% v/v FCS/DMEM. Serially diluted, renatured recombinant TGF β ₁ fusion proteins were added to the wells and incubated for 24 hours. ³H-thymidine (1 μ Ci/well, specific activity 2 Ci/mmol, 74 GBq/mmol, ICN) was added during the last 4 hours of incubation. Human platelet-derived TGF β ₁ (R & D Systems) was used as a standard and as a control. After incubation, the cells were precipitated twice with cold 10% (w/v) trichloroacetic acid (TCA),

25
30

extracted with 0.2 M NaOH, and neutralized with 0.5 M acetic acid for analysis by liquid scintillation counting in cocktail.

Mv1Lu cells were also used to assess the biological activity of the recombinant TGF β_1 -F1 fusion protein pre-absorbed onto collagen- and fibronectin-coated dishes. In these studies, 20 μ l of pepsin-treated, acid-extracted bovine tendon collagen type I or human plasma fibronectin (20 μ g/well; Telio) were dried onto each well of a 24-well plate (Costar) overnight. Following a brief ultraviolet light treatment to crosslink the matrix proteins, plates were counter-coated with 0.2% (w/v) bovine serum albumin (BSA). Serial dilutions of the recombinant TGF β_1 -F1 were added to the coated wells in PBS and incubated at 37°C for 2 hours. The wells were rinsed 3 times with DMEM, and Mv1Lu cells (1.5×10^5 per well in 0.1% (v/v) FCS/DMEM) were added to each well. The cells were incubated for about 20 hours at 37°C, followed by quantification of 3 H-thymidine incorporation into TCA-precipitable material as described above.

15

Example 10

Collagen Binding Assay

Two different approaches were used to assess the affinity of the recombinant TGF β_1 -F2 fusion protein for collagen and for gelatin.

20 In the first method, collagen and gelatin covalently conjugated to CNBr-activated Sephadex G-15 columns were used as test matrices. Preparations of native and denatured purified rat tail type I collagen were coupled onto CNBr-activated Sephadex G-15 beads. The beads were then washed extensively with 50 mM Tris buffer, pH 8, containing 1 M NaCl. The fusion protein was applied to the medium in a buffered saline solution and was eluted with a linear salt gradient from 0.2 to 1 M NaCl.

25 In the second method, the recombinant fusion protein was first immobilized onto Ni-NTA medium, then collagen (biosynthetically labeled with 3 H-proline and purified from human fibroblast cultures) was applied to the Ni-NTA medium, and 30 eluted with a linear gradient of either phosphate buffered NaCl (0.15 to 1.5 M) or urea (0 to 4 M).

Virtually, all of the recombinant growth factor bound to the collagen G-15 under these conditions, as determined by protein assays. Attempts to elute the bound protein from the collagen medium with a salt gradient or 2 M urea were ineffective, suggesting that $\text{TGF}\beta_1$ -F2 associates tightly with collagen. Similar results were
5 obtained when $\text{TGF}\beta_1$ -F2 was bound to a gelatin-Sephadex medium.

A different strategy was attempted in which the $\text{TGF}\beta_1$ -F2 fusion protein was first immobilized on Ni-NTA medium and then exposed to biosynthetically-labeled ^3H -collagen, which was loaded subsequently onto the medium. Under these conditions, a large portion of the radioactivity was found to bind to the medium.
10 Washing the medium with a linear gradient of NaCl from 0.15 to 1.5 M did not release the ^3H -collagen. However, application of a urea gradient (0 to 4.0 M) was able to quantitatively elute all bound radioactivity. In contrast, when the $\text{TGF}\beta_1$ -F1 fusion protein, comprising the $(\text{His})_6$ tag and the $\text{TGF}\beta_1$ active fragment, was applied to the Ni-NTA medium under identical conditions, very little ^3H -collagen was
15 retained on the medium, suggesting that the auxiliary collagen binding domain in $\text{TGF}\beta_1$ -F2 afforded this high affinity interaction.

Example 11

Stimulation of the Proliferation of NIH-3T3 Mouse Fibroblasts

20 The ability of $\text{TGF}\beta_1$ -F1 and $\text{TGF}\beta_1$ -F2 to stimulate the proliferation of NIH-3T3 mouse fibroblasts was assayed by plating NIH-3T3 mouse fibroblasts in 24 well plates at subconfluent densities (1.5×10^5 cells/well) and culturing the cells for 48 hours in DMEM containing 0.5% (v/v) fetal calf serum. Commercial $\text{TGF}\beta_1$ or
25 renatured $\text{TGF}\beta_1$ -F2 samples were then added to each well and incubated for 18 hours prior to the addition of ^3H -thymidine, followed by an additional 4 hours of incubation. To evaluate the effect of collagen-bound $\text{TGF}\beta_1$ -F2 on the 3T3 fibroblasts, the fusion protein was first bound to collagen-coated wells. Cells (1.5×10^5 cells/well) were seeded on top of the collagen with DMEM containing 0.5%
30 (v/v) fetal calf serum or 0.5% (w/v) ITS (insulin, transferrin and selenium from Collaborative Biomedical Products, MA), harvested by trypsinization 72 hours later and quantified by direct counting.

As observed with human platelet $\text{TGF}\beta_1$, $\text{TGF}\beta_1$ -F2 treatment of 3T3 fibroblasts for 18 hours following a 48-hour low serum starvation, resulted in a 30-fold increase in ^3H -thymidine incorporation. When $\text{TGF}\beta_1$ -F2 was applied and bound to collagen-coated culture wells, and then cells were seeded on top of the

5 $\text{TGF}\beta_1$ -F2/collagen in 0.5% (v/v) fetal calf serum/DMEM or ITS, no significant amount of stimulation of cell proliferation was observed, suggesting that the tight binding of $\text{TGF}\beta_1$ -F2 to collagen lowered the availability and/or the rate of release of the biologically active growth factor. By contrast, commercial $\text{TGF}\beta_1$ absorbed onto collagen-coated wells stimulated the proliferation of 3T3 cells approximately

10 fifteen fold.

Example 12

Evaluation of $\text{TGF}\beta$ /Collagen Matrices in Wound Healing Models

15 The function of the fusion $\text{TGF}\beta$ were assessed in a series of classic bone healing models. Comparative studies utilizing the rat calvarial defect model in which a collagen matrix is used in the presence or absence of adsorbed $\text{TGF}\beta$ fusion proteins. The rate and extent of bone healing will be evaluated by radiologic and histologic methods.

20 $\text{TGF}\beta_1$ fusion protein was found to have a profound effect on bone healing in the rat calvarial defect model. The results demonstrated a marked stimulation of wound closure and osteogenesis at 2 and 4 weeks after surgery. In control rats, scar tissue formation was observed, whereas in the $\text{TGF}\beta_1$ treated rats bone formation, rather than scar tissue was observed.

25 Histological examination of the tissue showed a marked recruitment of osteogenic precursors, a characteristic profile of cellular maturation and effective absorption of the original collagen matrix. By contrast, the collagen matrix alone (control) produced granulation tissue characterized by an infiltration of inflammatory cells. Quantification of calcium deposition in control versus $\text{TGF}\beta$ impregnated

30 collagen matrices revealed significant improvements which were evident within 2 weeks. Angiogenesis was evident with $\text{TGF}\beta$ /collagen, as was a remarkable absence of adhesions between the newly formed bone and the underlying dura mater.

Example 13Development and Characterization of a
Mesenchymal Stem Cell Trap

A diagram of wound healing stages observed within TGF β treated collagen
5 matrices depicts three major features: (I) recruitment and expansion of a
mesenchymal stem cell (MSC) population, (II) elaboration (of factors) and
differentiation of cellular phenotype, and (III) resolution and remodeling of the
extracellular matrix (see FIG. 1).

Based on the magnitude and extent of precursor cell migration and
10 proliferation observed in the rat calvarial model (Stage I), TGF β impregnated
collagen matrices were tested for their ability to selectively reinforce the proliferation
of mesenchymal stem cells that are present in low abundance within human bone
marrow aspirates under conditions that the remainder of the cellular components of
the marrow would not survive.

15 Rescue and selection of TGF β responsive stem cells from human bone
marrow aspirates upon 15 days of serum deprivation was observed.

Example 142-Stage Histogenesis of Human "Artificial Skin"
on Collagen Supports

20

Collagen matrices and sheets, though optimal in terms of structural integrity
and biodegradability, can cause inflammatory responses (rejection) and fibrosis
(scarring). By contrast, TGF β impregnated collagen matrices inhibit inflammatory
processes while promoting angiogenesis and histogenesis. TGF β is a natural and
25 critical component regulating epithelial-mesenchymal interactions in the
developmental morphogenesis of skin appendages. Collagen bound TGF β_1 -F2 fusion
proteins can function effectively to select and expand (capture) a population of
mesenchymal stem cells *in vitro*.

The development of an autologous "artificial" skin is feasible. The
30 experimental procedure is to select and expand a population of explanted human
fibroblasts, along with other resident mesenchymal precursors, within TGF β
impregnated collagen sheets. This procedure is continued *in vitro* up to an optimized
point whereby the collagen sheet is effectively cellularized yet not degraded. At or

just prior to this point, the collagen/connective tissue sheet is epithelialized by the application of an explanted plug of keratinocytes.

The human artificial skin comprised of TGF β impregnated collagen sheets which have been cellularized and epithelialized in a 2-Stage approach is evaluated histologically and, once experimental conditions have been optimized, the "skin" is tested in a nude mouse wound healing model.

In these animal models, several considerations and critical parameters will be evaluated, as follows: Comparative studies are performed to evaluate the outcome and scar formation involving TGF β /collagen sheets cultured under high serum conditions (mostly fibroblastic cells) versus TGF β /collagen sheets in which the population of pluripotent mesenchymal stem cells have been selected and expanded by culture under low serum conditions.

Such studies are expected to result in an enrichment (recruitment and expansion) of pluripotent stem cells will facilitate normal histogenesis and wound healing. The application of recombinant TGF β fusion proteins, including collagen-binding (F2) and fibronectin-binding (F3) constructs, to the cellularized "skin" (i.e., cellularized/epithelialized collagen sheets) and/or the wound surface is evaluated in terms of efficacy in promoting adherence, angiogenesis, and histogenesis. Such studies are also expected to result in a secondary application of TGF β fusion proteins which will retard rejection and promote fusion of cultured tissues. The timing of each stage of the *ex vivo* tissue culture, as well as the thickness and physicochemistry of the collagen sheets, is also assessed.

Example 15

The use of TGF- β as a Wound Healing Enhancing Agent

TGF β is known to effect wound healing by modulating stem cells proliferation and the expression of specific genes including those encoding for extracellular matrix proteins and cellular receptors. Numerous studies using animal models have demonstrated the potential of TGF β to promote wound healing. TGF β of the present invention exhibits biological activity and its use, in place of the naturally occurring TGF β , allows the treatment of many conditions which previously

have been considered as only potential clinical applications, due to the limited availability of TGF β .

Regeneration of the skeleton:

5 It has been found that injections of TGF β_1 on the periosteal layer stimulated bone formation in newborn rats. Different dosages of TGF β added to demineralized bone matrix paste, formed into cylinders and implanted onto the periosteum of rabbits have shown that TGF β induced higher levels of (accelerated) trabecular bone formation than controls. TGF β also caused greater resorption of the demineralized
10 bone. It has also been shown that a single application of TGF β_1 in a simple 3% methylcellulose gel to large skull defects in rabbits was able to induce intramembranous bone formation and complete bony bridging of defects was observed within 28 days after treatment of 2 μ g of TGF β . In neonatal rats, 12-day treatment of TGF β_1 injection onto the outer periosteum of the right side of the parietal
15 bone increased the number of osteoprogenitor cells, resulting in intramembranous ossification. In adult rats, TGF β_1 induced differentiation of chondrocytes. Cartilage masses were found to be surrounded by mesenchymal cells. In these animals the cartilage matrix was partially calcified, with chondrocytes buried therein. Marrow cavities containing some multinuclear osteoclasts were also observed in the calcified
20 matrix. These findings indicate that TGF β_1 stimulated the differentiation of mesenchymal cells into chondrocytes and produced cartilaginous matrix. TGF β_1 induced intramembranous ossification of the parietal bone in neonatal rats, and it induced endochondral ossification in adults. These results show different responses of mesenchymal cells in the periosteum to TGF β_1 which may depend on the age of
25 the animals used; namely, they may reflect the respective osteogenic stages of modeling and remodeling.

It has also been shown, that TGF β counteracts the deleterious effects of interleukin-1 (IL-1) on articular cartilage proteoglycan synthesis and content indicating that TGF β plays an important role in articular cartilage restoration after
30 IL-1 induced proteoglycan depletion. It has also been demonstrated that short term systemic injection of recombinant TGF β_2 increases cancellous bone formation rate in juvenile and adult rats. It has also been shown that continuous local application

of TGF β for 6 weeks enhances fracture healing of tibial defects in rabbits. Similarly, it has been shown that local injection of TGF β at the site of tibial fractures induced a dose-dependent increase in the cross-sectional area of the callus and bone at the fracture line.

5

Skin wound healing:

The treatment of incisional wounds of rats, which received total body radiation, with a single dose of TGF β_1 (2 μ g/wound) using 3% methylcellulose as a delivery vehicle, resulted in a significant acceleration of soft tissue repair and wound-breaking strength in the absence of monocytes and macrophages. TGF β_1 was not able to reverse healing deficit in the megavoltage electron beam surface irradiated skin wounds. The treatment of partial-thickness wounds in pigs topically with TGF β using Silvadene cream (Marion Labs, Kansas, Mo) as a vehicle was shown to accelerate the regeneration of dermis. It has also been shown that TGF β accelerated the maturation of a neo-vascularized skin flap in rabbits.

15

Protection and rescue from impaired wound healing:

It has been shown that mice which received TGF β prior to treatment of high doses of 5-fluoro-uracil exhibited a hematologic recovery and were preferentially rescued by a suboptimal number of transplanted bone marrow cells. It was also shown that pretreatment of mice with TGF β protected 70-80% of them from lethal doses of the noncycle active chemotherapeutic drug, doxorubicin hydrochloride (DXR). It has also been found that parenteral steroids (β -methasone, 12 mg/50 g injected intramuscularly twice daily) induced an impairment of breaking load on a healed longitudinal intestinal wound in pigs. TGF β in a collagen suspension was used to treat these wounds and was found to reverse the effect of the steroids and significantly strengthened these wounds.

25

Other studies have been directed at the effect of TGF β on an Adriamycin-impaired wound healing model. In this model, a systemic adriamycin injection (8 mg/kg) produces significant decreases in wound tear strength and wound tear energy when compared with that of normal rats at seven and 10 days. A single dose of TGF β (2 μ g) in a collagen vehicle was shown to stimulate a reversal of this wound

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healing impairment at day 10. Similarly, intravenously administered TGF β at 100-500 mg/kg dosage can reverse age- or glucocorticoid-impaired healing of incisional wounds. Treatment of experimental allergic encephalomyelitis (EAE) with TGF β_2 resulted in the inhibition of T-cell activation and proliferation *in vitro*. Long-term treatment was effective in reducing clinical severity of EAE suggesting a potential use of TGF β_2 as a therapeutic agent for human demyelinating diseases such as multiple sclerosis.

Protection against myocardial dysfunction and stroke:

10 It has been shown that TGF α reduces endothelial cell release of nitric oxide, while TGF β appears to protect against myocardial dysfunction induced by prolonged ischemia and reperfusion probably by reducing plasma TGF α levels, blocking neutrophil adherence, and promoting nitric oxide release.

Other studies have been directed at the effect of TGF β on thromboembolic stroke in a rabbit model. An autologous clot embolus was introduced intracranially through the right internal carotid artery of rabbits to induce a thromboembolic stroke. TGF β in an albumin vehicle was administered as an intracarotid bolus immediately before autologous clot embolization. The results showed treatment with 10 and 50 μ g TGF β reduced the infarct size and there was a greater return of cerebral blood flow in the first 2 hours after embolization. Other studies have addressed the ability of TGF to preserve endothelial functions of coronary arteries in dogs by infusing TGF into the left anterior descending coronary artery distal to the site subjected to multiple brief occlusions and reperfusion. TGF β_1 prevented impaired endothelium-dependent relaxation after multiple brief occlusions and reperfusions suggesting that TGF β_1 can play a protective role in the endothelial injury induced by repeated episodes of coronary artery occlusion and reperfusion.

Immune suppression:

It has also been shown that prolonged survival of cardiac graft transplants can be achieved by injecting plasmid DNA encoding TGF under the control of SV40 promoter into grafts from syngeneic or allogenic donors prior to implantation into recipients. In other studies in mice, the intramuscular injection of a vector encoding TGF β_1 depressed the anti-transferrin antibody response and caused an 8-fold increase in plasma TGF β_1 activity. The TGF β_1 plasmid injection induced biological effects characteristic of TGF β in regulating humoral and cellular immune responses *in vivo* but did not cause muscle infiltration with monocytes or neutrophils and there was no evidence for fibrotic changes.

Applications for humans currently under clinical trials:

Other studies have shown in a randomized multicenter clinical study of patients with full-thickness macular holes, that 0.66 pg of TGF β_2 , applied locally, to be successful in flattening the rim of subretinal fluid surrounding macular holes. The study further showed that TGF β_2 retreatment (1.33 pg) on full-thickness macular holes which failed to close after vitreous surgery appeared to have a beneficial effect on both neurosensory retinal flattening and visual outcome.

20 Pharmacokinetics of TGF β :

Other studies have included a detailed pharmacokinetic and tissue distribution study of TGF as a potential intravenous bolus or topical wound healing enhancing agent. It has been found that the half-life of topically administered TGF has a plasma half-life ranging between 61 to 163 minutes depending on the dose and duration of the treatment. Other studies have shown that ^{125}I TGF was detectable 16 days after a single dose of TGF, formulated in a 3% methylcellulose vehicle, in the rabbit calvarial defect model. It has also been demonstrated that high-dose dermal application of TGF resulted in local effects attributed to known biological activities of TGF β at the wound sites without systemic toxicity.

Example 16Methods of Delivery of TGF- β Systemic injection:

A suitable method to administer TGF β is injection of the growth factor in a
5 liquid vehicle.

Injection of DNA vector:

Plasmid DNA encoding the human TGF β_1 under the control of a known
promoter can be injected intramuscularly.

10

Topical application:

Silvadene cream (3% methylcellulose) and soluble collagen are useful as
vehicles in topical or local administration of TGF β .

15 Implantable solid phase carriers:

TGF β (1 to 10 μ g) enclosed in a gelatin capsule containing methylcellulose
can be implanted into surgical chambers and in bone to increased bone formation.
Biodegradable controlled release systems for TGF β_1 which comprise poly (DL-lactic-
co-glycolic acid) (PLPG) and demineralized bone matrix (DBM) can be used. DBM
20 alone, 3% methylcellulose gel, and alginate beads are also effective carriers for
TGF β .

The present invention is not to be limited to the specific embodiments which
are shown or described above and which are merely illustrative. Various and
numerous other arrangements and applications may be devised by one skilled in the
25 art without departing from the spirit and scope of this invention. For example, one
skilled in the art will be aware that the DNA sequences can be changed without
changing the amino acid sequence of the proteins encoded or that the DNA can be
changed to change the amino acid specified at a particular place in a polypeptide, but
which do not change the functional properties of the polypeptide produced from the
30 DNA sequence, i.e. conservative substitutions. Such modified DNA sequences and

amino acid sequences are considered to be included within the scope of the present invention.

The scope of the invention is defined in the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Hall, Frederick L.
Nimni, Marcel E.
Tuan, Tai-Lan
Wu, Lintao
Cheung, David T.
- (ii) TITLE OF INVENTION: Transforming Growth Factor B Fusion and
Their Use in Wound Healing
- (iii) NUMBER OF SEQUENCES: 34
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Merchant & Gould
 - (B) STREET: 11150 Santa Monica Boulevard, Suite 400
 - (C) CITY: Los Angeles
 - (D) STATE: California
 - (E) COUNTRY: USA
 - (F) ZIP: 90025-3395
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/470,837
 - (B) FILING DATE: June 6, 1995
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Sharp, Janice A.
 - (B) REGISTRATION NUMBER: 34,051
 - (C) REFERENCE/DOCKET NUMBER: 30774.1W001

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 310-445-1140
- (B) TELEFAX: 310-445-9031

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

His Asp Val Leu Lys

1

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Leu Val Tyr

1

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Ala Pro Phe

1

- (2) INFORMATION FOR SEQ ID NO:4:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Pro Ala

1

- (2) INFORMATION FOR SEQ ID NO:5:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Ala Ala

1

- (2) INFORMATION FOR SEQ ID NO:6:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Arg

1

- (2) INFORMATION FOR SEQ ID NO:7:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Thr Arg

1

- (2) INFORMATION FOR SEQ ID NO:8:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Pro Ile Glu Phe Phe Arg Leu

1

5

- (2) INFORMATION FOR SEQ ID NO:9:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Pro Ala Lys Phe Phe Arg

1

5

- (2) INFORMATION FOR SEQ ID NO:10:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Ser Phe Leu Ala Leu
1 5

- (2) INFORMATION FOR SEQ ID NO:11:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Ala Phe
1

- (2) INFORMATION FOR SEQ ID NO:12:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: cDNA
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..12
 - (ix) FEATURE:
 - (A) NAME/KEY: mat_peptide
 - (B) LOCATION: 1
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATC GAA GGT CGT
12
Ile Glu Gly Leu
1

- (2) INFORMATION FOR SEQ ID NO:13:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 4 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ile Glu Gly Leu

1

- (2) INFORMATION FOR SEQ ID NO:14:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: cDNA
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..18
 - (ix) FEATURE:
 - (A) NAME/KEY: mat_peptide
 - (B) LOCATION: 1
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTG GTT CCG CGT GGA TCC

18

Leu Val Pro Arg Gly Ser

1

5

- (2) INFORMATION FOR SEQ ID NO:15:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Leu Val Pro Arg Gly Ser

1

5

38

- (2) INFORMATION FOR SEQ ID NO:16:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Arg Gly Asp

1

- (2) INFORMATION FOR SEQ ID NO:17:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: cDNA
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..18
 - (ix) FEATURE:
 - (A) NAME/KEY: mat_peptide
 - (B) LOCATION: 1
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGT GGC TGG AGC CAC TGG

18

Gly Gly Trp Ser His Trp

1

5

- (2) INFORMATION FOR SEQ ID NO:18:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Gly Gly Trp Ser His Trp

1

5

- (2) INFORMATION FOR SEQ ID NO:19:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: cDNA
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..27
 - (ix) FEATURE:
 - (A) NAME/KEY: mat_peptide
 - (B) LOCATION: 1
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGG CGC GAA CCG AGC TTC ATG GCT CTG
27
Trp Arg Glu Pro Ser Phe Met Ala Leu
1 5

- (2) INFORMATION FOR SEQ ID NO:20:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Trp Arg Glu Pro Ser Phe Met Ala Leu
1 5

- (2) INFORMATION FOR SEQ ID NO:21:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: cDNA
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..18
 - (ix) FEATURE:
 - (A) NAME/KEY: mat_peptide
 - (B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAT CAT CAT CAT CAT CAC

18

His His His His His His

1

5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

His His His His His His

1

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(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..45

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AAA GAA ACC GCT GCT GCT AAA TTC GAA CGC CAG CAC ATG GAC AGC

45

Lys Glu Thr Ala Ala Ala Lys Phe Glu Arg Gln His Met Asp Ser

1

5

10

15

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Lys Glu Thr Ala Ala Ala Lys Phe Glu Arg Gln His Met Asp Ser
 1 5 10 15

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 654 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..654

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATG TCC CCT ATA CTA GGT TAT TGG AAA ATT AAG GGC CTT GTG CAA CCC 48
 Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val Gln Pro
 1 5 10 15

ACT CGA CTT CTT TTG GAA TAT CTT GAA GAA AAA TAT GAA GAG CAT TTG 96
 Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu
 20 25 30

TAT GAG CGC GAT GAA GGT GAT AAA TGG CGA AAC AAA AAG TTT GAA TTG 144
 Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu
 35 40 45

GGT TTG GAG TTT CCC AAT CTT CCT TAT TAT ATT GAT GGT GAT GTT AAA 192
 Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys
 50 55 60

TTA ACA CAG TCT ATG GCC ATC ATA CGT TAT ATA GCT GAC AAG CAC AAC 240
 Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn
 65 70 75 80

ATG TTG GGT GGT TGT CCA AAA GAG CGT GCA GAG ATT TCA ATG CTT GAA 288
 Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu
 85 90 95

WO 96/39430

42

GGA GCG GTT TTG GAT ATT AGA TAC GGT GTT TCG AGA ATT GCA TAT AGT 336
 Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser
 100 105 110
 AAA GAC TTT GAA ACT CTC AAA GTT GAT TTT CTT AGC AAG CTA CCT GAA 384
 Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu
 115 120 125
 ATG CTG AAA ATG TTC GAA GAT CGT TTA TGT CAT AAA ACA TAT TTA AAT 432
 Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn
 130 135 140
 GGT GAT CAT GTA ACC CAT CCT GAC TTC ATG TTG TAT GAC GCT CTT GAT 480
 Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp
 145 150 155 160
 GTT GTT TTA TAC ATG GAC CCA ATG TGC CTG GAT GCG TTC CCA AAA TTA 528
 Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
 165 170 175
 GTT TGT TTT AAA AAA CGT ATT GAA GCT ATC CCA CAA ATT GAT AAG TAC 576
 Val Cys Phe Lys Lys Arg Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr
 180 185 190
 TTG AAA TCC AGC AAG TAT ATA GCA TGG CCT TTG CAG GGC TGG CAA GCC 624
 Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
 195 200 205
 ACG TTT GGT GGT GGC GAC CAT CCT CCA AAA 654
 Thr Phe Gly Gly Gly Asp His Pro Pro Lys
 210 215

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 218 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val Gln Pro
 1 5 10 15
 Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu
 20 25 30

43

Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu
 35 40 45
 Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys
 50 55 60

 Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn
 65 70 75 80

 Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu
 85 90 95

 Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser
 100 105 110

 Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu
 115 120 125

 Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn
 130 135 140

 Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp
 145 150 155 160

 Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
 165 170 175

 Val Cys Phe Lys Lys Arg Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr
 180 185 190

 Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
 195 200 205

 Thr Phe Gly Gly Gly Asp His Pro Pro Lys
 210 215

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..27

44

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TAC CCA TAC GAT GTT CCA GAT TAC GCT

27

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala

1

5

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala

1

5

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 339 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..333

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..336

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GCC CTG GAC ACC AAC TAT TGC TTC AGC TCC ACG GAG AAG AAC TGC TGC
Ala Leu Asp Thr Asn Tyr Cys Phe Ser Ser Thr Glu Lys Asn Cys Cys

1

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15

48

45

GTG CGG CAG CTG TAC ATT GAC TTC CGC AAG GAC CTC GGC TGG AAG TGG 96
 Val Arg Gln Leu Tyr Ile Asp Phe Arg Lys Asp Leu Gly Trp Lys Trp
 20 25 30

ATC CAT GAG CCC AAG GGC TAC CAT GCC AAC TTC TGC CTC GGG CCC TGC 144
 Ile His Glu Pro Lys Gly Tyr His Ala Asn Phe Cys Leu Gly Pro Cys
 35 40 45

CCC TAC ATT TGG AGC CTG GAC ACG CAG TAC AGC AAG GTC CTG GCC CTG 192
 Pro Tyr Ile Trp Ser Leu Asp Thr Gln Tyr Ser Lys Val Leu Ala Leu
 50 55 60

TAC AAC CAG CAT AAC CCG GGC GCC TCG GCG GCG CCG TGC TGC GTG CCG 240
 Tyr Asn Gln His Asn Pro Gly Ala Ser Ala Ala Pro Cys Cys Val Pro
 65 70 75 80

CAG GCG CTG GAG CCG CTG CCC ATC GTG TAC TAC GTG GGC CGC AAG CCC 288
 Gln Ala Leu Glu Pro Leu Pro Ile Val Tyr Tyr Val Gly Arg Lys Pro
 85 90 95

AAG GTG GAG CAG CTG TCC AAC ATG ATC GTG CGC TCC TGC AAG TGC AGC 336
 Lys Val Glu Gln Leu Ser Asn Met Ile Val Arg Ser Cys Lys Cys Ser
 100 105 110

TGA

339

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 112 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Ala Leu Asp Thr Asn Tyr Cys Phe Ser Ser Thr Glu Lys Asn Cys Cys
 1 5 10 15

Val Arg Gln Leu Tyr Ile Asp Phe Arg Lys Asp Leu Gly Trp Lys Trp
 20 25 30

Ile His Glu Pro Lys Gly Tyr His Ala Asn Phe Cys Leu Gly Pro Cys
 35 40 45

Pro Tyr Ile Trp Ser Leu Asp Thr Gln Tyr Ser Lys Val Leu Ala Leu
 50 55 60

46

Tyr Asn Gln His Asn Pro Gly Ala Ser Ala Ala Pro Cys Cys Val Pro
65 70 75 80

Gln Ala Leu Glu Pro Leu Pro Ile Val Tyr Tyr Val Gly Arg Lys Pro
85 90 95

Lys Val Glu Gln Leu Ser Asn Met Ile Val Arg Ser Cys Lys Cys Ser
100 105 110

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 339 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..336

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCT TTG GAT GCG GCC TAT TGC TTT AGA AAT GTG CAG GAT AAT TGC TGC
48

Ala Leu Asp Ala Ala Tyr Cys Phe Arg Asn Val Gln Asp Asn Cys Cys
1 5 10 15

CTA CGT CCA CTT TAC ATT GAT TTC AAG AGG GAT CTA GGG TGG AAA TGG
96

Leu Arg Pro Leu Tyr Ile Asp Phe Lys Arg Asp Leu Gly Trp Lys Trp
20 25 30

ATA CAC GAA CCC AAA GGG TAC AAT GCC AAC TTC TGT GCT GGA GCA TGC 144
Ile His Glu Pro Lys Gly Tyr Asn Ala Asn Phe Cys Ala Gly Ala Cys
35 40 45

CCG TAT TTA TGG AGT TCA GAC ACT CAG CAC AGC AGG GTC CTG AGC TTA 192
Pro Tyr Leu Trp Ser Ser Asp Thr Gln His Ser Arg Val Leu Ser Leu
50 55 60

TAT AAT ACC ATA AAT CCA GAA GCA TCT GCT TCT CCT TGC TGC GTG TCC 240
Tyr Asn Thr Ile Asn Pro Glu Ala Ser Ala Ser Pro Cys Cys Val Ser
65 70 75 80

AAG ATT GAA CAG CTT TCT AAT ATG ATT GTA AAG TCT TGC AAA TGC AGC 336
Lys Ile Glu Gln Leu Ser Asn Met Ile Val Lys Ser Cys Lys Cys Ser
100 105 110

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 112 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Leu Arg Pro Leu Tyr Ile Asp Phe Lys Arg Asp Leu Gly Trp Lys Trp
20 25 30

Pro Tyr Leu Trp Ser Ser Asp Thr Gln His Ser Arg Val Leu Ser Leu
50 55 60

Gln Asp Leu Glu Pro Leu Thr Ile Leu Tyr Tyr Ile Gly Lys Thr Pro
85 90 95

Lys Ile Glu Gln Leu Ser Asn Met Ile Val Lys Ser Cys Lys Cys Ser
100 105 110

48

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 339 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..336

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

| | |
|---|-----|
| GCT TTG GAC ACC AAT TAC TGC TTC CGC AAC TTG GAG GAG AAC TGC TGT | 48 |
| Ala Leu Asp Thr Asn Tyr Cys Phe Arg Asn Leu Glu Glu Asn Cys Cys | |
| 1 5 10 15 | |
| GTG CGC CCC CTC TAC ATT GAC TTC CGA CAG GAT CTG GGC TGG AAG TGG | 96 |
| Val Arg Pro Leu Tyr Ile Asp Phe Arg Gln Asp Leu Gly Trp Lys Trp | |
| 20 25 30 | |
| GTC CAT GAA CCT AAG GGC TAC TAT GCC AAC TTC TGC TCA GGC CCT TGC | 144 |
| Val His Glu Pro Lys Gly Tyr Tyr Ala Asn Phe Cys Ser Gly Pro Cys | |
| 35 40 45 | |
| CCA TAC CTC CGC AGT GCA GAC ACA ACC CAC AGC ACG GTG CTG GGA CTG | 192 |
| Pro Tyr Leu Arg Ser Ala Asp Thr Thr His Ser Thr Val Leu Gly Leu | |
| 50 55 60 | |
| TAC AAC ACT CTG AAC CCT GAA GCA TCT GCC TCG CCT TGC TGC GTG CCC | 240 |
| Tyr Asn Thr Leu Asn Pro Glu Ala Ser Ala Ser Pro Cys Cys Val Pro | |
| 65 70 75 80 | |
| CAG GAC CTG GAG CCC CTG ACC ATC CTG TAC TAT GTT GGG AGG ACC CCC | 288 |
| Gln Asp Leu Glu Pro Leu Thr Ile Leu Tyr Tyr Val Gly Arg Thr Pro | |
| 85 90 95 | |
| AAA GTG GAG CAG CTC TCC AAC ATG GTG GTG AAG TCT TGT AAA TGT AGC | 336 |
| Lys Val Glu Gln Leu Ser Asn Met Val Val Lys Ser Cys Lys Cys Ser | |
| 100 105 110 | |
| TGA | 339 |

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 112 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Ala Leu Asp Thr Asn Tyr Cys Phe Arg Asn Leu Glu Glu Asn Cys Cys
1 5 10 15

Val Arg Pro Leu Tyr Ile Asp Phe Arg Gln Asp Leu Gly Trp Lys Trp
20 25 30

Val His Glu Pro Lys Gly Tyr Tyr Ala Asn Phe Cys Ser Gly Pro Cys
35 40 45

Pro Tyr Leu Arg Ser Ala Asp Thr Thr His Ser Thr Val Leu Gly Leu
50 55 60

Tyr Asn Thr Leu Asn Pro Glu Ala Ser Ala Ser Pro Cys Cys Val Pro
65 70 75 80

Gln Asp Leu Glu Pro Leu Thr Ile Leu Tyr Tyr Val Gly Arg Thr Pro
85 90 95

Lys Val Glu Gln Leu Ser Asn Met Val Val Lys Ser Cys Lys Cys Ser
100 105 110

WHAT IS CLAIMED IS:

1. A transforming growth factor- β fusion protein comprising:
a purification tag; and
a transforming growth factor active fragment.
- 5 2. A transforming growth factor- β fusion protein as recited in claim 1
further comprising at least one proteinase site.
3. A transforming growth factor- β fusion protein as recited in claim 1
further comprising an extracellular matrix binding site.
- 10 4. A transforming growth factor- β fusion protein as recited in claim 1
wherein the purification tag is selected from the group consisting of a (His)_n tag, a
ribonuclease S tag, a schistosoma japonicum glutathione S-transferase tag or a
hemagglutinin A tag.
- 15 5. A transforming growth factor- β fusion protein as recited in claim 1
wherein the purification tag is selected from the group consisting of the sequence of
SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:28 or SEQ ID NO:28.
- 20 6. A transforming growth factor- β fusion protein as recited in claim 4
wherein n is 6.
7. A transforming growth factor- β fusion protein as recited in claim 2
wherein the proteinase site is selected from the group consisting of a thrombin
25 cleavage site, a Factor Xa cleavage site, a plasmin cleavage site, a chymotrypsin
cleavage site, an elastase cleavage site, a trypsin cleavage site, a pepsin cleavage
site, a chymosin cleavage site or a thermolysin cleavage site.

8. A transforming growth factor- β fusion protein as recited in claim 2 wherein the proteinase site is selected from the group consisting of the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:13 or SEQ ID NO:15.

9. A transforming growth factor- β fusion protein as recited in claim 3 wherein the extracellular matrix binding site is selected from the group consisting of a fibronectin binding site, a collagen binding site or a cell surface binding site.

10. A transforming growth factor- β fusion protein as recited in claim 3 wherein the extracellular matrix binding site is selected from the group consisting of the sequence of SEQ ID NO:16, SEQ ID NO:18 or SEQ ID NO:20.

11. A transforming growth factor- β fusion protein as recited in claim 1 wherein the transforming growth factor active fragment is selected from the group consisting of the active fragment of TGF β_1 , TGF β_2 or TGF β_3 .

12. A transforming growth factor- β fusion protein as recited in claim 1 wherein the transforming growth factor active fragment is selected from the group consisting of the sequence of SEQ ID NO:30, SEQ ID NO:32 or SEQ ID NO:34.

13. A method of preparing a transforming growth factor- β fusion protein comprising:

isolating a protein fraction comprising mature transforming growth factor- β fusion protein;

denaturing the protein fraction in denaturation buffer;

purifying the mature transforming growth factor- β fusion protein from the protein mixture by a process comprising:

contacting the mature transforming growth factor- β fusion protein with a metal chelate resin;

washing the mature transforming growth factor- β fusion protein/metal chelate resin with denaturation buffer, pH 8.0 to remove non-specifically bound material;

5 washing the mature transforming growth factor- β fusion protein/metal chelate resin with denaturation buffer, pH 6.5 to remove additional non-specifically bound material; and

washing the mature transforming growth factor- β fusion protein/metal chelate resin with denaturation buffer, pH 4.0 to elute mature transforming growth factor- β fusion protein;

10 diluting the mature transforming growth factor- β fusion protein solution to a concentration of 0.05 to 0.5 mg/ml with a buffer comprising 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.05% (v/v) NP-40, 2 mM reduced glutathione, 0.2 mM oxidized glutathione;

15 storing the diluted mature transforming growth factor- β fusion protein solution for about 16 hours at 4°C;

dialyzing the diluted mature transforming growth factor- β fusion protein solution against an equal volume of dialysis buffer for about 20 minutes;

replacing the dialysis buffer with twice the volume of dialysis buffer as used in the preceding step;

20 repeating the step of replacing the dialysis buffer until the volume of the dialysis buffer is equal to about 10 times the volume of the diluting the mature transforming growth factor- β fusion protein solution;

storing the dialysis for about 16 hours at about 4°C without stirring;

stirring the dialysis for about 30 minutes;

25 replacing the dialysis buffer;

stirring the dialysis for about 2 hours; and

collecting the dialysate.

14. A method of preparing a transforming growth factor- β fusion protein
30 as recited in claim 13 wherein the denaturation buffer comprises 8 M urea, 0.1 M sodium phosphate, 10 mM Tris, pH 8.0.

15. A method of preparing a transforming growth factor- β fusion protein as recited in claim 14 wherein the denaturation buffer further comprises a reducing agent.

5 16. A method of preparing a transforming growth factor- β fusion protein as recited in claim 15 wherein the reducing agent is β -mercaptoethanol.

17. A method of preparing a transforming growth factor- β fusion protein as recited in claim 13 wherein the dialysis buffer comprises 20 mM Tris, pH 8.0,
10 250 mM NaCl, 20% (v/v) glycerol.

18. A method of preparing a transforming growth factor- β fusion protein as recited in claim 13 further comprising digesting the purified transforming growth factor- β fusion protein with a proteinase to cleave the transforming growth factor- β
15 fusion protein at a proteinase site.

19. A method of preparing a transforming growth factor- β fusion protein as recited in claim 18 further comprising purifying a transforming growth factor- β fragment from the digested transforming growth factor- β fusion protein.

I/I

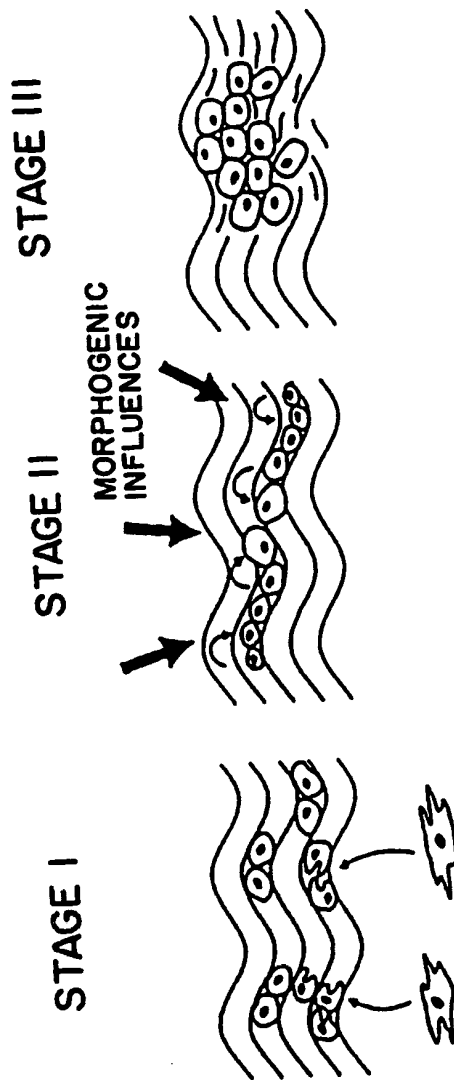


FIG. I

INTERNATIONAL SEARCH REPORT

International Application No
PC/US 96/08973

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/495 C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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G document member of the same patent family

Date of the actual completion of the international search

17 October 1996

Date of mailing of the international search report

05. 11. 96

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Groenendijk, M

INTERNATIONAL SEARCH REPORT

International Application No
PC 1/US 96/08973

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